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The 90-kDa Heat Shock Protein (hsp-90) Possesses an ATP Binding Site and Autophosphorylating Activity*

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The 90-kDa heat shock protein (hsp-90) is an abundant cytosolic protein believed to play a role in maintenance of protein trafficking and closely associated with several steroid hormone receptors. Incubation of highly purified hsp-90 with [γ-^{32}P]ATP results in its autophosphorylation on serine residues. There are several lines of evidence which suggest that this activity is due to a kinase intrinsic to hsp-90 rather than some closely associated protein kinases: 1) the phosphorylation persists after the removal of casein kinase II by heparin chromatography and after immunoprecipitation of hsp-90 with anti-hsp-90 antibodies. 2) The approximate k_M for ATP of the reaction is 0.16 mM, higher than that of many other protein kinases. 3) Phosphorylation is not affected by a number of activators and inhibitors of other known kinases which might associate with hsp-90. 4) The phosphorylation displays a unique cation dependence being most active in the presence of Ca^{2+} and practically inactive with Mg^{2+}, although the autophosphorylation in the presence of Mg^{2+} is activated by histones and polyamines. 5) The activity is remarkably heat-stable; incubation of hsp-90 for 20 min at 95 °C results in only a 60% decrease in autophosphorylation. 6) Finally, and most importantly, purified hsp-90 can be labeled with azido-ATP and it is able to bind to ATP-agarose. The binding shows similar cation dependence to the autophosphorylation. These data are in agreement with the presence of a consensus sequence for ATP binding sites in the primary structure of the protein similar to that observed in the 70-kDa heat-shock proteins. Our data suggest the 90-kDa heat shock protein possesses an enzymatic activity analogous in many respects to the similar activity of the 70-kDa heat shock proteins. This may represent an important, previously unrecognized function of hsp-90.

Heat shock proteins are a set of predominantly cytoplasmic proteins universally expressed in organisms in response to temperatures above their natural range and to various other environmental stresses (1-4). The major heat shock proteins can be grouped into three size classes based on their approximate molecular weights and degrees of homology. One class is composed of proteins with molecular size between 15 and 30 kDa; members of the second and most highly conserved group have molecular sizes of approximately 70 kDa; and the third class consists of heat shock proteins with molecular sizes ranging between 80 and 105 kDa.

A member of the latter group, the 90-kDa heat shock protein (hsp-90),[1] is present in most, if not all, prokaryotic and eukaryotic cells and may constitute up to 1-2% of the total cytosolic protein. hsp-90 is associated with most steroid hormone receptors in their non-ligand-bound state and is believed to act as a "molecular chaperone" preventing the premature association of steroid receptors with DNA (2-5). hsp-90 also forms complexes with a number of protein kinases, including casein kinase II, double-stranded DNA-activated protein kinase, heme-regulated eIF-2α kinase, protein kinase C, and various tyrosine kinases (4, 6-12). In addition, hsp-90 associates with the microfilamental and microtubular network, raising the possibility that it may use these cellular trajectories in targeting the steroid receptors and other proteins to the nucleus (13-15). Despite this information, the exact function of hsp-90 is incompletely elucidated.

In the present report we present evidence that hsp-90 possesses an ATP binding site and the ability to phosphorylate itself on serine residues. These findings represent the first demonstration of an apparent enzyme activity associated with this abundant cellular protein and may help to understand its function in normal and stressed cells.

EXPERIMENTAL PROCEDURES

Chemicals—Butyl-Sepharose 4B, DEAE-Sepharose Fast Flow, and Sepharose S-200 resins were purchased from Pharmacia LKB Biotechnology Inc. Spectrogel HA hydroxylapatite resin was from Spectrum Medical Industries (Los Angeles, CA). Bio-Gel P-6DG resin and all the chemicals used for polyacrylamide gel electrophoresis were from Bio-Rad. The rabbit (AC88 and D7a) and sheep anti-hsp-90 antibodies were kindly provided by Drs. David O. Toft (Mayo Medical School, Rochester, MN) and Michael J. Welsch (University of Michigan, Medical School, Ann Arbor, MI), respectively. AC88 recognizes "free" hsp-90, *i.e.* hsp-90 which is not complexed with steroid hormone receptors, whereas D7a and the goat anti-hsp-90 antibodies react with both the free and "bound" hsp-90 (16, 17). Anti-phosphotyrosine antibodies were a gift of Dr. Morris F. White (Joslin Diabetes Center). Protein A-acrylamide beads, constant boiling 6 N HCl, and Triton X-100 were obtained from Pierce Chemical Co. The tyrosine kinase inhibitor tyrphostin (18) was kindly provided by Dr. Joseph Schles-

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[1] The abbreviations used are: hsp-90, 90-kDa heat shock protein; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenebis-(oxyethylenenitrilo)]tetraacetic acid; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

singer (Rorer Biotechnology, King of Prussia, PA). [γ-^{32}P]ATP (111 TBq/mmol), and [γ-^{32}P]GTP (111 TBq/mmol) were from Du Pont-New England Nuclear; [α-^{32}P]8-azido-ATP (366 GBq/mmol) was from ICN Biomedicals Inc. (Irvine, CA). TPCK-trypsin was obtained from Worthington. All the other chemicals used were from Sigma.

Isolation of hsp-90—The 90-kDa heat shock protein was isolated from livers of 2–3 month-old, male Sprague-Dawley rats using the method of Yonezawa *et al.* (19) with the following modifications: in all buffers Hepes was used instead of Tris; ATP (at a final concentration of 1 mM) was included in the buffers of the butyl-Sepharose 4B and DEAE-Sepharose Fast Flow chromatographies; a gel filtration step was added using a Sepharose S-200 (60 × 2 cm) column in between the DEAE and hydroxylapatite ion exchange chromatographies. hsp-90 was detected by immunoblotting with sheep anti-hsp-90 antibody. After the Spectragel HA hydroxylapatite chromatography the purified hsp-90 was dialyzed against 4 liters of 50 mM Hepes, pH 7.4, 0.5 mM DTT buffer. The purity of this preparation was higher than 95% (usually higher than 98%) as judged by densitometry of Coomassie Blue-stained SDS slab gels. A significant portion of the small amount of contaminating proteins were derived from proteolysis of hsp-90 and were recognized by the anti-hsp-90 antibody D7a in immunoblots. For some experiments, an even more highly purified preparation of hsp-90 was prepared in which any trace amounts of casein kinase II potentially associated with hsp-90 were removed by heparin affinity chromatography (20). This preparation is referred to as "highly purified hsp-90" or heparin-purified hsp-90.

Phosphorylation and Dephosphorylation of hsp-90—5 μg of the appropriate hsp-90 preparation was incubated in the presence of 50 mM Hepes, pH 7.4, 200 μM of [γ-^{32}P]ATP or [γ-^{32}P]GTP (5,000–6,000 and 10,000–12,000 cpm/pmol, respectively) and 10 mM of the indicated divalent cation for 20 min at 30 °C. The reaction was stopped by boiling for 5 min in the presence of Laemmli buffer (21) containing 10 mM EDTA, and 20 mM DTT. Samples were analyzed by SDS-PAGE and autoradiography. The radioactivity of the hsp-90 bands was quantitated by densitometry of the autoradiograms and by direct Cerenkov counting of the bands in 20% methanol.

In some experiments hsp-90 was prephosphorylated before labeling with [^{32}P]ATP. 1 mg of hsp-90, or "heparin-purified" hsp-90, was incubated for 30 min at room temperature in the presence of 50 mM Hepes, pH 7.4, 2 mM ATP, and 5 mM Mg^{2+} or Ca^{2+}, respectively; the excess of ATP was removed by gel filtration on a Bio-Gel P-6DG column. In other experiments hsp-90 was dephosphorylated before [^{32}P]ATP labeling. For these, 0.5 mg of heparin-purified hsp-90 was incubated with 30 units of alkaline phosphatase immobilized on agarose in the presence of 50 mM Hepes, pH 8.0, and 5 mM $MgCl_2$ at room temperature with continuous rotation. After 2 h, the buffer was changed to 50 mM Hepes, pH 7.4, and the sample was concentrated using Centricon 30 ultrafiltrators.

Covalent Labeling of hsp-90 with [α-^{32}P]Azido-ATP—5 μg of hsp-90 was preincubated with 4 μM (1 μCi) [α-^{32}P]azido-ATP in the dark in separate wells of a 96-well microtiter plate at 4 °C for 15 min in a buffer containing 50 mM Hepes, pH 7.4, and 10 mM of the indicated divalent cation. The reaction mixture was irradiated with a 100-watt long wavelength UV lamp (Black Ray, UVP Inc., San Gabriel, CA) for 5 min at 4 °C from a distance of 10 cm. Samples were transferred to Eppendorf microcentrifuge tubes containing 30 μl of Laemmli buffer (21) supplemented with 10 mM EDTA and 20 mM DTT, boiled for 5 min, and analyzed by SDS-PAGE and autoradiography. The radioactivity of the hsp-90 bands was quantitated by densitometry of the autoradiograms and by liquid scintillation counting.

Immunoprecipitation of hsp-90—hsp-90 was immunoprecipitated with rabbit (AC88 and D7a) or sheep anti-hsp-90 antibodies. The reaction mixtures of the phosphorylation or 8-azido-ATP labeling experiments (containing 5 μg of hsp-90) were incubated overnight with 10 μg of the affinity purified antibodies at 4 °C in the presence of 10 mM ATP, or in the dark, respectively. Samples were diluted to 0.5 ml with 50 mM Hepes, pH 7.4, and 30 μl of protein A-acrylamide beads was added. After 2 h of rotation at 4 °C, samples were centrifuged and successively washed with 1 ml each of buffers containing 50 mM Hepes, pH 7.4, supplemented with 0.1% (w/v) SDS, 1% (v/v) Triton X-100, and 0.1% (v/v) Triton X-100, respectively. The final pellets were eluted with Laemmli buffer (21) containing 20 mM DTT and analyzed by SDS-PAGE and autoradiography.

Phosphoamino Acid Analysis—Phosphoamino acid analysis was carried out by the method of Cooper *et al.* (22). hsp-90 bands were excised from the acrylamide gel, soaked in 20% methanol, digested with the addition of 2 × 100 μg of TPCK-trypsin in 50 mM ammonium carbonate for 24 h at 37 °C. The tryptic digest was lyophilized and hydrolyzed in constant boiling 6 N HCl at 110 °C for 60 min. The hydrolysates were washed with 2 × 1 ml of distilled water and subjected to electrophoresis on TLC plates at pH 3.5. Plates were dried, stained with ninhydrin, and analyzed by autoradiography.

Other Methods—ATPase activity was determined in a 0.1-ml reaction medium containing 5 μg of hsp-90, 0.5 mM [γ-^{32}P]ATP (200,000 cpm/sample), 5 mM of the indicated divalent cation, and 50 mM Hepes, pH 7.4, or 50 mM Tris, pH 8.4. The amount of [^{32}P] inorganic phosphate was determined by extraction of its phosphomolybdate complex to organic phase and liquid scintillation counting (23). ATP-dependent protease activity was measured by the detection of the hydrolysis of fluorescein isothiocyanate-labeled casein (24). The reaction mixture contained 5 μg of hsp-90, 2 mg/ml fluorescein isothiocyanate-labeled casein, 50 mM Hepes, pH 7.4, 0.5 mM DTT, with or without 5 mM ATP and 5 mM $MgCl_2$ or $CaCl_2$. Samples were incubated at 30 °C for 60 min, precipitated with trichloroacetic acid, and the fluorescence of the neutralized supernatants was measured (24). Protein concentrations were determined using the method of Bradford (25) and bovine γ-globulin as standard.

RESULTS

Phosphorylation of hsp-90—Incubation of heparin affinity chromatography purified hsp-90 with 0.2 mM [γ-^{32}P]ATP and 5 mM Ca^{2+} resulted in heavy labeling of the 90-kDa hsp band (Fig. 1, *lane a*). This label persisted after immunmoprecipitation with anti-hsp-90 antibody (*lane c*). In the presence of excess ATP (10 mM) the labeling was decreased by about 80% (*lanes b* and *d*). Thus, highly purified hsp-90 appeared to undergo autophosphorylation catalyzed by a saturable enzymatic process. Neither ^{32}P-labeled inorganic phosphate nor [α-^{32}P]ATP was able to label hsp-90 (data not shown), thereby excluding the possibility that the observed effect was due to either tight association of radioactive ATP with some highly charged regions of hsp-90 or poly-(ADP-ribosyl)ation of the protein (26). By contrast, [γ-^{32}P]GTP induced similar phosphorylation of hsp-90 as [γ-^{32}P]ATP (Fig. 1, *lanes e–h*). When analyzed at multiple concentrations of nucleotide triphosphate, the k_M of the reaction was 0.16 mM for ATP and 0.44 mM for GTP (Fig. 2).

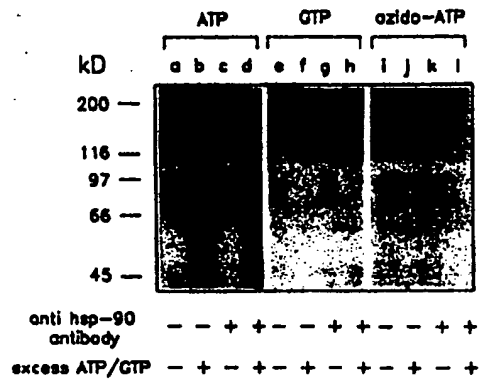

FIG. 1. Autophosphorylation and azido-ATP labeling of hsp-90. 5 μg of heparin affinity purified hsp-90 was phosphorylated by 200 μM [γ-^{32}P]ATP (*lanes a–d*) or [γ-^{32}P]GTP (*lanes e–h*) in the presence of 10 mM Ca^{2+} for 20 min at 30 °C as described under "Experimental Procedures." Samples represented in *lanes b, d, f,* and *h* were phosphorylated in the presence of 10 mM ATP or 10 mM GTP, respectively. In separate experiments, 5 μg of heparin affinity purified hsp-90 was labeled with 4 μM [α-^{32}P]azido-ATP (*lanes i–l*) in the presence of 10 mM Ca^{2+} by illuminating with UV light for 5 min at 4 °C as described under "Experimental Procedures." Samples represented by *lanes j* and *l* were labeled in the presence of 10 mM ATP. After the phosphorylation or labeling, some samples were immunoprecipitated with the rabbit anti-hsp-90 antibody D7a as described under "Experimental Procedures" (*lanes c, d, g, h, k,* and *l*). Samples were subjected to SDS-PAGE and autoradiography. The autoradiogram shown is representative of three separate experiments.

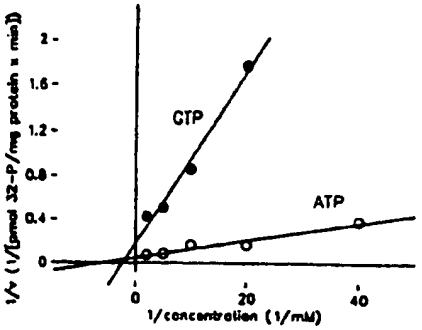

FIG. 2. Double-reciprocal plot of the ATP and GTP dependence of hsp-90 autophosphorylation. 5 μg of heparin affinity purified hsp-90 was phosphorylated by various concentrations of [γ-^{32}P]ATP (O——O) or [γ-^{32}P]GTP (●- - -●) in the presence of 10 mM Ca^{2+} for 20 min at 30 °C as described under "Experimental Procedures." Preliminary experiments indicated that the autophosphorylation was a linear function of time up to 30 min in the concentration range tested. Samples were analyzed by SDS-PAGE and the amount of incorporated ^{32}P was calculated by measuring the radioactivity of the hsp-90 bands. Data represent the mean of two separate experiments.

Since it is well known that the 90-kDa heat shock protein forms complexes with a number of serine, threonine, and tyrosine protein kinases (4, 6–12), we examined the effect of known inhibitors and activators of these enzymes on the phosphorylation of hsp-90. The phosphorylation of hsp-90 in the presence of Mg^{2+} was strongly inhibited by both heparin and 5,6-dichlorobenzimidazole riboside suggesting that casein kinase II was present at this step of purification. By contrast, the phosphorylation of heparin affinity purified hsp-90 in the presence of Ca^{2+} was not significantly affected by 50 μg/ml double-stranded DNA, 5 μg/ml heparin, 5 mM 5,6-dichlorobenzimidazole riboside, 200 μM hemin, 100 μM H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine, 100 μg/ml phosphatidylserine plus 10 μg/ml diacylglycerol, and 100 μM of the tyrosine kinase inhibitor tyrphostin (18) which modify the activity of the double-stranded DNA-activated protein kinase, casein kinase II, heme-regulated eIF-2α kinase, protein kinase C, and various tyrosine kinases, respectively (4, 6–12, 18; data not shown). Thus, it seemed unlikely that a contaminating protein kinase was responsible for the observed phosphorylation of hsp-90.

Covalent Labeling of hsp-90 with [α-^{32}P]8-Azido-ATP—Observing the phosphorylation of hsp-90 in highly purified preparations of the protein and gaining some evidence that none of the kinases known to be associated with hsp-90 seem to be involved in this process, we examined whether hsp-90 could be covalently labeled with radioactive azido-ATP. Following incubation and UV radiation of hsp-90 with the ATP affinity reagent the 90-kDa band was heavily labeled. This labeled band was immunoprecipitated with anti-hsp-90 antibodies (Fig. 1, *lanes i* and *k*). ATP (10 mM) effectively competed for labeling by radioactive azido-ATP in both the non-immunoprecipitated and immunoprecipitated samples (*lanes j* and *l*).

Binding of hsp-90 to ATP-Agarose—In addition to labeling with azido-ATP, 30 and 76% of the heparin affinity purified hsp-90 was able to bind to ATP-agarose in the presence of Ca^{2+} and Mn^{2+}, respectively, both ions which support autophosphorylation (Table I, and see below). The binding of hsp-90 was negligible in the presence of Mg^{2+}, without divalent cations or without covalently attached ATP. These results provide further evidence that hsp-90 has a nucleotide binding site.

TABLE I

Binding of hsp-90 to ATP-agarose

50 μg of purified hsp-90 was applied to a 50-μl ATP-agarose (A4793, Sigma) or agarose (A0169, Sigma) microcolumn equilibrated with a buffer containing 50 mM Hepes, pH 7.4, and 10 mM of the divalent cation indicated. The column was washed with 4 × 0.2 ml of the same buffer, and the bound hsp-90 was eluted with 4 × 0.2 ml of Hepes, pH 7.4, 10 mM EDTA solution. The amount of hsp-90 was quantified by determination of protein content of each fraction collected. The percentage of hsp-90 bound represents the relative amount of protein in the EDTA elution as a percentage of total hsp-90 recovered. Data are the mean of two separate experiments.

hsp-90 in the presence of	% bound
Mg^{2+}-(ATP-agarose)	5
Ca^{2+}-(ATP-agarose)	30
Mn^{2+}-(ATP-agarose)	76
EDTA, EGTA-(ATP-agarose)	4
Mn^{2+}-(agarose)	10

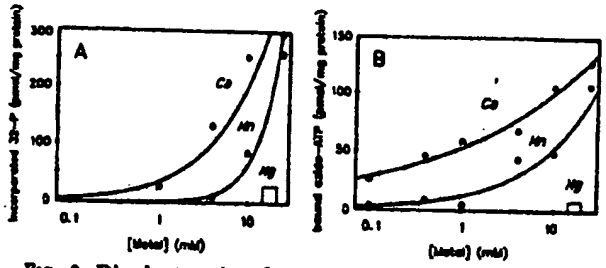

FIG. 3. Divalent cation dependence of the autophosphorylation and azido-ATP labeling of hsp-90. 5 μg of heparin affinity purified hsp-90 was phosphorylated by 200 μM [γ-^{32}P]ATP for 20 min at 30 °C (*A*) or labeled with 4 μM [α-^{32}P]azido-ATP using 5-min UV illumination at 4 °C (*B*) as described under "Experimental Procedures." The phosphorylation or labeling was carried out in the presence of Ca^{2+} (*filled circles*) or Mn^{2+} (*open circles*) at indicated final concentrations. The *bars* represent experiments done in the presence of 25 mM Mg^{2+}. Samples were analyzed by SDS-PAGE. The amount of incorporated ^{32}P or bound azido-ATP was calculated by measuring the radioactivity of the hsp-90 bands. Data represent the mean of two separate experiments.

Metal Dependence of the Phosphorylation and Azido-ATP Labeling of hsp-90—The autophosphorylation of hsp-90 was supported by Ca^{2+} and, to a lesser extent, by Mn^{2+} at final concentrations higher than 1 mM (Fig. 3*A*). The azido-ATP labeling of heparin affinity purified hsp-90 displayed similar dependence on Ca^{2+} and Mn^{2+} (Fig. 3*B*). In contrast, there was virtually no autophosphorylation or azido-ATP labeling in the presence of Mg^{2+} even at final concentrations as high as 25 mM (Fig. 3, *A* and *B*). Neither the phosphorylation nor the azido-ATP labeling reached saturation in the concentration range tested. In our experiments, however, some precipitation of the samples occurred if the concentration of the divalent cations was raised above 25 mM; therefore, we did not extend the concentration range above this value. Calmodulin, a modulator of the association of hsp-90 with actin (14), did not induce any significant changes in the level of hsp-90 phosphorylation in the presence of either micromolar or millimolar Ca^{2+} (data not shown). From the data of Fig. 3 it can be calculated that under our normal assay conditions (10 mM Ca^{2+} and 0.2 mM ATP), 1–2% of the total hsp-90 became phosphorylated or covalently labeled after 20 min at 30 °C using an hsp-90 concentration of 0.1 mg/ml.

The metal dependence of the phosphorylation of hsp-90 in standard hsp-90 preparations displayed a markedly different pattern than the highly purified hsp-90. For "standard" hsp-90, Mg^{2+} was the most active among the three divalent cations tested, while Ca^{2+} and Mn^{2+} induced a much slighter effect

(data not shown). This different behavior most probably reflects the presence of some casein kinase II in the less purified preparations which readily phosphorylates hsp-90 in the presence of Mg-ATP (20).

Histones, and Protamine, but Not Polyamines Activate hsp-90 Phosphorylation in the Presence of Magnesium—Autophosphorylation of hsp-90 was modified by polycations depending on the divalent metal ion used for activation. Lysine-rich histone lowered the phosphorylation of heparin affinity purified hsp-90 in the presence of calcium, had no effect in the presence of manganese, and activated several-fold the phosphorylation in the presence of magnesium (Fig. 4). The effect of lysine-rich histones on the calcium-induced autophosphorylation of hsp-90 varied in different experiments. In most of the experiments lysine-rich histone lowered the autophosphorylation only slightly (Table II, column "Ca^{2+}"); however, in one of the experiments (shown in Fig. 4) the inhibition was almost complete. In the presence of Mg^{2+}-lysine-rich histones produced a more pronounced activation of hsp-90 autophosphorylation than the arginine-rich fractions (Table II, column "Mg^{2+}"). Type II-S histone, a mixture of lysine- and arginine-rich fractions, displayed an intermediate activity. Protamine showed similar characteristics as lysine-rich histone. These effects, however, are not a general feature of polycations since polyamines did not change the phosphorylation of hsp-90 (Table II, column "Mg^{2+}"). This latter observation also argues against the possibility that the magnesium-dependent kinase activity is due to the activation of a contaminating kinase by histones or protamine, since the kinase most likely associated with hsp-90, casein kinase II, is also activated by polyamines (27, 28). The assumption that histones and protamine activate the same kinase activity which is displayed at the Ca^{2+}-dependent phosphorylation of hsp-90 is further supported by the fact that this Mg^{2+}-dependent activity is also heat-stable (data not shown).

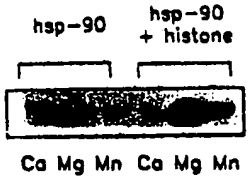

FIG. 4. **The effect of lysine-rich histone on the divalent cation dependence of hsp-90 autophosphorylation.** The autophosphorylation of 5 μg of heparin affinity purified hsp-90 was carried out in the absence or presence of 5 μg of lysine-rich histone (Type III-S, Sigma) with 10 mM final concentration of the indicated divalent cation and 200 μM [γ-^{32}P]ATP for 20 min at 30 °C as described under "Experimental Procedures." After phosphorylation samples were subjected to SDS-PAGE and autoradiography. The autoradiogram shown is representative of three separate experiments.

TABLE II

Autophosphorylation of hsp-90 in the presence of various basic substances

The phosphorylation of heparin affinity purified hsp-90 was carried out as described under "Experimental Procedures" in the absence or presence of 5 μg of various basic substances and 10 mM of Ca^{2+} or Mg^{2+} as indicated. Samples were analyzed by SDS-PAGE, the 90-kDa bands of the gels were cut out, and the amount of their radioactivity was quantified by liquid scintillation counting. Data are representative of three separate experiments.

	^{32}P incorporated	
	Ca^{2+}	Mg^{2+}
	pmol/mg protein	
Control	120	10
+Histone (type II-S)	93	50
+Histone (Arg-rich)	82	30
+Histone (Lys-rich)	80	150
+Protamine	110	120
+Putrescine	115	13
+Spermidine	110	10
+Spermine	119	11

Heat Stability of hsp-90 Phosphorylation—The effect of a 10-min preincubation at various temperatures on the activity of hsp-90 in the presence of Mg^{2+}, Mn^{2+}, and Ca^{2+} is shown in Fig. 5*A*. The magnesium-dependent activity (which was most probably derived from casein kinase II since it could be inhibited by the inhibitors of this enzyme) displayed a typical heat inactivation pattern for many enzymes, being almost completely inactivated at 55 °C within 10 min. However, the Mn^{2+}- and Ca^{2+}-dependent phosphorylation of hsp-90 was extremely heat-stable with a significant amount of the activity remaining after a 10-min preincubation at 95 °C. Furthermore, the Ca^{2+}- and Mn^{2+}-dependent autophosphorylation of hsp-90 persisted even after 20 min of preincubation at 95 °C, while the Mg^{2+}-dependent activity was eliminated after 2 min (Fig. 5*B*). In the experiments shown, the preincubations were carried out in the absence of the divalent cations. Similar data were obtained if the preincubations were done in the presence of the indicated cations. The heparin-purified hsp-90 preparation in the presence of Ca^{2+} and Mn^{2+} displayed identical heat inactivation pattern as the less purified hsp-90, although in this case Mg^{2+}-dependent phosphorylation was too low to obtain a reasonable pattern of heat inactivation (data not shown).

Phosphoamino Acid Analysis—The phosphorylation of heparin affinity purified hsp-90 occurred predominantly on phosphoserine both in the presence of Ca^{2+} (Fig. 6*A*) or Mg^{2+} plus lysine-rich histone (Fig. 6*B*). These results are in agreement with our other experiments which showed that phosphorylated hsp-90 was not immunoprecipitated with anti-phosphotyrosine antibodies and that the phosphate group(s) of hsp-90 is acid-stable but alkali-sensitive (data not shown). The acid stability of the label also argues against the presence of any phospholysine, phosphoarginine, or phosphohistidine residues (29). Polyacrylamide gel electrophoresis at pH 2.0 (30) did not produce any significant increase in the amount of radioactive label recovered, suggesting no alkaline-sensitive acyl-phosphate intermediates (data not shown).

Effect of Pre- and Dephosphorylation on the Autophosphorylation of hsp-90—The prephosphorylation of heparin affin-

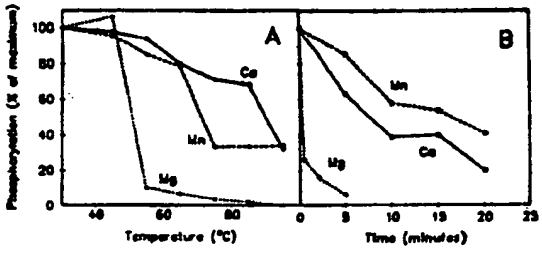

FIG. 5. **Heat stability of hsp-90 phosphorylation.** 5 μg of hsp-90 was incubated in 50 mM Hepes buffer, pH 7.4, for 10 min at indicated temperatures (*A*) or at 95 °C for indicated times (*B*). After heat treatment, samples were cooled to 30 °C and phosphorylated with 200 μM [γ-^{32}P]ATP in the presence of 10 mM Ca^{2+} (*open circles*), 10 mM Mn^{2+} (*filled circles*), or 10 mM Mg^{2+} (*stars*) for 20 min as described under "Experimental Procedures." After phosphorylation samples were analyzed by SDS-PAGE. The radioactivity of the hsp-90 bands was measured and expressed as a percentage of control sample which had a heat treatment at 30 °C for 10 min. "100% phosphorylation" was 580, 150, and 60 pmol of ^{32}P/mg protein in the presence of Mg^{2+}, Ca^{2+}, and Mn^{2+}, respectively. Data represent the mean of two separate experiments.

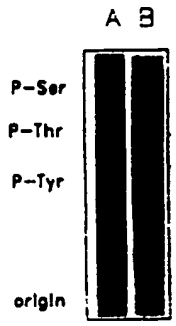

FIG. 6. **Phosphoamino acid analysis of hsp-90.** 5 μg of heparin affinity purified hsp-90 was phosphorylated by 200 μM [γ-^{32}P]ATP in the presence of 10 mM Ca^{2+} (*A*) or 5 μg of lysine-rich histone and 10 mM Mg^{2+} (*B*) for 20 min at 30 °C and subjected to SDS-PAGE as described under "Experimental Procedures." The 90-kDa hsp-90 band was excised, digested by 2 × 100 μg of TPCK-trypsin at 37 °C for 24 h, hydrolyzed in 6 N HCl at 110 °C for 60 min, and subjected to high voltage electrophoresis at pH 3.5 on a TLC plate. Plates were dried, stained with ninhydrin, and analyzed by autoradiography. The autoradiogram shown is a representative of three separate experiments.

TABLE III

Effect of prephosphorylation and dephosphorylation on the autophosphorylation and azido-ATP labeling of hsp-90

Heparin affinity purified hsp-90 was phosphorylated or labeled with azido-ATP without any previous treatment ("Control"), after prephosphorylation in the presence of 10 mM Ca^{2+}, or after dephosphorylation by immobilized alkaline phosphatase as described under "Experimental Procedures." In the case of the Mg^{2+}-prephosphorylated hsp-90, hsp-90 was first phosphorylated in the presence of 10 mM Mg^{2+} and then subjected to heparin affinity chromatography and successive phosphorylation or azido-ATP labeling. After phosphorylation or labeling with azido-ATP samples were analyzed by SDS-PAGE, the 90-kDa bands of the gels were cut out, and the amount of their radioactivity was quantified by liquid scintillation counting. Data are the mean of two (in the case of "Dephosphorylated hsp-90," four) separate experiments.

	^{32}P-incorporated		Azido-ATP-bound	
	Ca^{2+}	Mn^{2+}	Ca^{2+}	Mn^{2+}
	pmol/mg protein			
Control	112	30	192	24
Ca^{2+}-prephosphorylated hsp-90	44	14	260	29
Mg^{2+}-prephosphorylated hsp-90	90	31	197	27
Dephosphorylated hsp-90	53	16	171	38

ity purified hsp-90 in the presence of Ca^{2+} diminishes the amount of radioactive label acquired in a subsequent incubation of the protein with Ca^{2+} and [γ-^{32}P]ATP (Table III). If we prephosphorylated hsp-90 in the presence of Mg^{2+} (where the phosphorylation was most probably induced by casein kinase II), the amount of autophosphorylation did not change significantly. However, if hsp-90 was dephosphorylated first with immobilized alkaline phosphatase, the subsequent radioactive phosphorylation was significantly diminished. A control experiment where only the buffer of alkaline phosphatase, but not the enzyme itself was added did not result in any significant changes in autophosphorylation (data not shown). Azido-ATP binding to hsp-90 was not significantly altered by any of these treatments (Table III).

hsp-90 Does Not Possess an ATPase- or ATP-dependent Protease Activity—The existence of a nucleotide binding site in the 90-kDa heat shock protein prompted us to speculate whether the protein might display ATP-dependent activities other than phosphorylation, such as ATPase or ATP-dependent protease activities. Since the 70-kDa heat shock proteins are ATPases (2–4), we examined whether hsp-90 was also able to hydrolyze ATP. hsp-90 showed no significant ATPase activity at pH 7.4 or at pH 8.5 in the presence of Mg^{2+}, Ca^{2+}, or Mn^{2+} (data not shown).

Nonlysosomal protein degradation utilizes mostly ATP-dependent proteases (31). Since in some of our preparations we observed some minor bands moving around 70–80 kDa which were recognized by anti-hsp-90 antibodies in immunoblots which may be products of some limited proteolysis, we examined whether hsp-90 had any ATP-dependent protease activity. However, hsp-90 did not cause any significant proteolysis of fluorescein isothiocyanate-labeled casein, a good substrate of ATP-dependent proteases (31), either in the absence or in the presence of ATP and Mg^{2+}, Ca^{2+}, or Mn^{2+} (data not shown).

DISCUSSION

The 90-kDa heat shock protein (hsp-90) is a highly conserved stress protein which is abundant in the cytoplasm of most prokaryotic and eukaryotic cells. Although the synthesis of hsp-90 is strongly induced by elevated temperatures and by various other stimuli, relatively high amounts of hsp-90 are also detected in unstressed cells (1–4). This has led to the hypothesis that hsp-90 has an important function in cells which becomes even more needed after injury or stress. Although the exact function of hsp-90 is uncertain, it has a tendency to form complexes with a variety of other proteins, including steroid receptors, protein kinases, actin, and tubulin (2–15). Our present knowledge is very much limited concerning the mechanism, consequences, and significance of these associations. One of the major missing links is that thus far no intrinsic activity other than binding of proteins has been assigned to hsp-90. The current study provides evidence that hsp-90 possesses a nucleotide binding site and may be a kinase able to autophosphorylate itself.

When incubated with [γ-^{32}P]ATP or [γ-^{32}P]GTP, highly purified preparations of hsp-90 become phosphorylated in a time-dependent manner. Since hsp-90 is known to be associated with a number of protein kinases (4, 6–12), one possible explanation of this phenomenon is that traces of contaminating kinases are still present in the hsp-90 and induce the phosphorylation of the 90-kDa band. Several lines of evidence show, however, that this is not the case:

1) The phosphorylation of hsp-90 persists after the removal of casein kinase II by heparin affinity chromatography and after immunoprecipitation of hsp-90 by anti-hsp-90 antibodies.

2) The approximate k_M of the reaction is 0.16 mM for ATP. This is much higher than the reported values for most other protein kinases and considerably higher than that for casein kinase II (6 μM) (28) or protein kinase C (1.5–15 μM) (32). Furthermore, phosphorylation is not affected by a number of activators and inhibitors of the known kinases which might associate with hsp-90.

3) The phosphorylation displays a unique cation dependence being most active in the presence of Ca^{2+}, moderately active in the presence of Mn^{2+} but practically inactive with Mg^{2+}.

4) The activity is remarkably heat-stable. hsp-90 retains significant autophosphorylation activity even after incubation for 20 min at 95 °C.

5) The phosphorylation is substrate-specific. Although hsp-90 is capable of autophosphorylation, thus far, in preliminary experiments, we have not observed a significant phosphorylation of histones, protamine, casein, phosvitin, actin, Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly), and angiotensin, well known substrates of the kinases usually associated with hsp-

90, by the purified hsp-90 preparation (data not shown).

Thus, the data strongly suggest that hsp-90 itself possesses an intrinsic autophosphorylating activity.

Consistent with this observation, hsp-90 possesses an ATP binding site. hsp-90 can be labeled with 8-azido-ATP and can bind to ATP-agarose. Examination of the primary sequence of the protein (33) and comparison of it to the consensus sequence for ATP binding sites in other heat shock proteins (34–37) reveals that hsp-90 contains homologous regions for both the "A" and "B" sequences associated with ATP binding (Table IV). Although this is a different type of ATP binding site than that of other serine or tyrosine kinases, it is highly homologous with the ATP binding sites of the 70-kDa heat shock proteins. hsp-90 can also utilize GTP for its autophosphorylation. GTP has a similar efficiency as ATP in competing with azido-ATP binding (data not shown). However, hsp-90 does not have an ideal sequence match with the GTP binding consensus sequence (38), and the k_M for GTP (0.44 mM) is too high to permit GTP as the preferred substrate over ATP *in vivo*. The almost identical metal dependence of the autophosphorylation and azido-ATP labeling further supports our conclusion that hsp-90 is a kinase capable of autophosphorylating itself.

The ability of hsp-90 to undergo autophosphorylation depends on the pre-existing phosphorylation state of the protein. The somewhat unexpected finding that dephosphorylation of hsp-90 actually decreases its successive autophosphorylation suggests that phosphorylation *in vivo* on a specific residue(s) may be necessary for the autophosphorylation reaction. A similar phenomenon has been observed in reticulocytes where hsp-90 is not able to stimulate the heme-sensitive eIF-2α kinase in its dephosphorylated form (39).

Under our experimental conditions relatively minor amounts (1–2%) of the total hsp-90 became phosphorylated. This extent of autophosphorylation is significantly smaller than the stoichiometric, or almost stoichiometric autophosphorylation of most protein kinases (40). However, in the case of the dnaK protein of *Escherichia coli*, which shows an extensive analogy with hsp-90 (see below), the extent of autophosphorylation is similarly low, 1–4% (41). It is also possible that only a subpopulation of hsp-90 (isoform, monomer, prephosphorylated hsp-90, or otherwise different hsp-90) is participating in the autophosphorylation and this subpopulation incorporates stoichiometric amounts of phosphate. Further experiments are needed to resolve this question.

The autophosphorylation and ATP binding of hsp-90 show many similarities with the properties of the 70-kDa heat shock proteins (hsp-70) and the related dnaK protein of *E. coli*. The hsp-70 family is highly conserved through evolution and displays an ATP binding consensus sequence similar to that of hsp-90. Likewise, the dnaK protein, which is highly homologous with the eukaryotic hsp-70 proteins, possesses a similar ATP binding sequence and is also able to autophosphorylate itself (41–43). Autophosphorylation of dnaK has properties almost identical to those we have observed for hsp-90. Both hsp-90 and dnaK have similar metal ion dependence for autophosphorylation with $Ca^{2+} > Mn^{2+} \gg Mg^{2+}$ (43). The concentration dependence of the autophosphorylation with Ca^{2+} is also similar (41, 43). Both proteins display a remarkable heat stability being still significantly active even after a treatment at 95 °C (41–43) both hsp-90 and the dnaK protein are able to utilize GTP as a substrate (41), and the overall fraction of the protein being autophosphorylated is similar, about 1–4% (41). The analogy, however, is not complete. hsp-90 autophosphorylates predominantly on serine, whereas dnaK phosphorylates on threonine. dnaK protein also possesses an ATPase activity which we could not detect in hsp-90. This latter difference might be anticipated by analyzing the primary structures of the two proteins. The ATPase activity of the dnaK protein has been found to be associated with its highly conserved amino-terminal portion which shows no homology whatever with hsp-90 (44).

One attractive hypothesis for a physiological function of hsp-90-associated kinase activity would relate to the association of hsp-90 with various steroid hormone receptors. Most steroid hormone receptors are multiply phosphorylated on serin residues, and hsp-90 is a component of the "nontransformed" (8 S) form of steroid receptors which is observed in the absence of ligand binding (2–5). About 10 years ago a calcium-dependent protein kinase activity was also reported

TABLE IV

ATP binding sequences of hsp-90 and related proteins

Protein	Residues	Sequence[a]
Type A sequence[b]		
Consensus sequence (34, 35)[c]		**A/G-X-X-X-X-G-K-T/S-X-X-X-X-X-X-I/V**
Rat hsp-70 (36)	131–144	**A-E-A-Y-L-G-K-T---V-T-N-A-V---V**
E. coli dnaK (37)	127–140	**A-E-D-Y-L-G-E-P---V-T-E-A-V---V**
	41–54	**A-Y-T-Q-D-G-E-T---L-V-G-Q-P-A**
Murine hsp-90-α (33)	534–549	**L-K-E-P-E-G-K-T---L-V-S-V-T-K-E-G**
hsp-90-β (33)	526–541	**L-K-E-P-D-G-K-S---L-V-S-V-T-K-E-G**
Type B sequence[b]		
Consensus sequence (34, 35)		**H/R/K---- $X_{(8-8)}$ ----ϕ-X-ϕ-ϕ-D/E**[d]
Rat hsp-70 (36)	56–69	**K-N-Q-V-A-M-N-P-T-N-T-V-F-D**
	187–199	**K-K-V-G-A-E-R-N---V-L-I-F-D**
	387–395	**K-S-G-N-V-Q-D-----L-L-L-L-D**
E. coli dnaK (37)	84–98	**R-D-V-S-I-M-P-F-K-I-I-A-A-D**
	151–164	**R-Q-A-T-K-D-A-G-R-I-A-G-L-E**
	447–460	**R-A-A-D-N-K-S-L-G-Q-F-N-L-D**
Murine hsp-90-α (33)	363–373	**K-L-Y-V-R-R-------V-F-I-M-D**
	513–524	**R-K-H-G-L-E-V-----I-T-M-I-E**
hsp-90-β (33)	354–364	**K-L-Y-V-R-R-------V-F-I-M-D**

[a] Gaps are marked with hyphens and consensus sequences are in boldface.
[b] "Type A" and "Type B" sequences denote the putative triphosphate and adenine binding sequences, respectively (34, 35).
[c] The numbers in parentheses denote the appropriate references.
[d] In the "Type B" consensus sequence "ϕ" stands for hydrophobic residues.

to be associated with the purified, nontransformed form of glucocorticoid and progesterone receptors (45–47), although subsequently one of these studies was retracted (48) and two other studies have shown no protein kinase activity associated with either the highly purified, "transformed" (4 S) form of the glucocorticoid receptor or with the associated 90-kDa protein (which turned out to be hsp-90 in the subsequent studies) (49, 50). Kost *et al.* (51) were also unable to show azido-ATP labeling of the progesterone receptor-associated hsp-90.

These latter observations seemingly contradict our results showing the autophosphorylation and azido-ATP binding of hsp-90. There are, however, several important differences between these studies and the current study. First, Sanchez and Pratt (49) and Hapgood *et al.* (50) studied the phosphorylation of hsp-90 primarily in the presence of Mg^{2+} which, under our assay conditions, does not support its activity. In their experiments using Ca^{2+} as divalent cation, molybdate was also present, and we find that molybdate inhibits the hsp-90-associated kinase activity (data not shown). Kost *et al.* (51) used Mn^{2+} as divalent cation in their experiments, a cation which moderately supports the azido-ATP binding and autophosphorylation of hsp-90. In these studies the source of the hsp-90 was from the nontransformed form of the steroid receptor complex, whereas in our studies hsp-90 was purified directly from liver cytosol. Since only a small fraction of hsp-90 undergoes autophosphorylation, it is also possible that this portion of the protein represents a different pool of hsp-90 from the one which has been dissociated from the steroid receptors. Furthermore, since prephosphorylation might enhance the autophosphorylation of hsp-90, it is possible that the hsp-90 derived from the nontransformed steroid receptor does not meet this condition. The dissociation of hsp-90 from steroid receptors and its autophosphorylation may be linked events. If this is indeed the case, then the *in vitro* autophosphorylation sites may already be occupied in the "steroid receptor-derived" hsp-90.

There are some correlations between the *in vitro* and *in vivo* phosphorylation of hsp-90 (2–5). Although autophosphorylation of hsp-90 *in vitro* requires relatively high concentrations of Ca^{2+}, histones (most probably the lysine-rich histone H1 and/or H2A) and protamine change the activation pattern in favor of Mg^{2+}. This raises the possibility that the autophosphorylation of hsp-90 might be linked to its association/encounter with other proteins, *e.g.* histones. This possibility is interesting since a portion of hsp-90 is found in the nucleus (52) which may be the place where steroid receptors dissociate from hsp-90 (52, 53). These considerations have led us to the hypothesis that histones may play a role in the dissociation of hsp-90 from steroid receptors which in turn may be linked with the autophosphorylation of hsp-90. To test this hypothesis, further studies are needed.

If hsp-90 is indeed a kinase, does it possess phosphotransferase activity or does it exclusively phosphorylate itself as has been suggested for the *ras* proteins (54)? Our preliminary experiments have thus far revealed no significant phosphorylation of histones, protamine, casein, phosvitin, actin, Kemptide, and angiotensin by hsp-90 (data not shown) and indicate that, if hsp-90 is a protein kinase, it must have a very limited substrate specificity. Our experiment showing no significant increase in the radioactive label after acidic gel electrophoresis argues against the formation of an acyl-phosphate intermediate which is a requirement in some phosphate transfer reactions. On the other hand the relatively high k_M of the reaction (which is similar to that of 3-phosphoglycerokinase, glycerol kinase, hexokinase, creatine kinase, or nucleotide kinases (55–59)) may suggest that hsp-90 phosphorylates substrates other than proteins.

In summary, our data indicate that hsp-90 possesses an autophosphorylating activity and a nucleotide binding site. This represents the first enzyme activity assigned to this abundant cellular polypeptide. Further studies of the autophosphorylation of hsp-90 may help us to understand the function of this protein in unstressed and stressed cells.

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